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Attestation

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Confirmation

It is hereby confirmed that the attached documents are corresponding with the original pages of the international application, as identified on the following pages, filed under Article 10 of the Patent Cooperation Treaty (PCT) at the receiving office named below.

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PCT REQUEST

159-16.WO-P

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0	For receiving Office use only	
0-1	International Application No.	PCT/CH 03 / 00568
0-2	International Filing Date	22. Aug. 2003 (22.08.03)
0-3	Name of receiving Office and "PCT International Application"	RO / CH - Internationale Anmeldung PCT
0-4	Form - PCT/RO/101 PCT Request	
0-4-1	Prepared using	PCT-EASY Version 2.92 (updated 01.07.2003)
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	Swiss Federal Intellectual Property Institute (RO/CH)
0-7	Applicant's or agent's file reference	159-16.WO-P
I	Title of invention	PLANT-DERIVED PEPTIDE HARBORING WATER-CLEANING AND ANTIMICROBIAL ACTIVITIES
II	Applicant	
II-1	This person is:	applicant only
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V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW MZ SD SL SZ TZ UG ZM ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE BG CH&LI CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL PT RO SE SI SK TR and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GQ GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH&LI CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NI NO NZ OM PG PH PL PT RO RU SC SD SE SG SK SL SY TJ TM TN TR TT TZ UA UG UZ VC VN YU ZA ZM ZW
V-5	**Precautionary Designation Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.	
V-6	**Exclusion(s) from precautionary designations**	NONE
VI	**Priority claim**	NONE
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)

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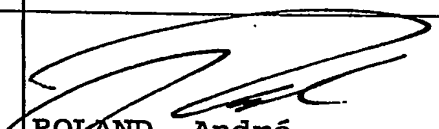
VIII	Declarations	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	Check list	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	4	-
IX-2	Description	47	-
IX-3	Claims	1	-
IX-4	Abstract	1	EZABST00.TXT
IX-5	Drawings	0	-
IX-7	TOTAL	53	
	Accompanying items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-17	PCT-EASY diskette	-	Diskette
IX-19	Figure of the drawings which should accompany the abstract		
IX-20	Language of filing of the international application	English	
X-1	Signature of applicant, agent or common representative		
X-1-1	Name (LAST, First)	ROLAND, André	

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10-1	Date of actual receipt of the purported international application	22. Aug. 2003 (22.08.03)
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10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	X

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11-1	Date of receipt of the record copy by the International Bureau	

Plant-Derived Peptide Harboring Water-Cleaning and Antimicrobial Activities

Summary

Drinking water is a world resource that is scarce. In many areas of the world where there is no access to water treatments plants, people employ traditional methods to clarify and disinfect water. The example that interest us cames from mixing cracked *Moringa oleifera* seeds with turbid water. Here I show the expression and analysis of a protein, called Flo (6.5 kDa, 60 amino acids, isoelectric point 11.61), identified in the seeds of *Moringa oleifera* tree. This protein has the capacity of sediment suspended mineral particles and bacteria, clarifying thus, turbid water. Furthermore, we also identified in Flo an efficient antimicrobial activity. Specifically, this peptide exhibits a bacteriostatic and bactericidal effect and it is effective against gram negative and gram positive bacteria. Sequence comparisons have revealed that Flo has homologies with 2S protein family. One of the members (napins) includes natural antifungal proteins. Since Flo displays a highly cationic structure, it may make part of the cationic antibiotic polypeptide group. To better understand the manner this peptide exerts its activity, we modelized Flo structure based on homologies with 2S seed proteins. This modelization showed three alpha helices on Flo sequence. Based on this result, several subfragments were synthesized with the aim of determine whether the whole Flo polypeptide is important for the full activity and whether common or distinct sequences are responsible of coagulation and disinfection activities. In the case of the coagulation activity, a 21 amino acid length fragment corresponding to the middle part of Flo efficiently replaces the full length Flo. In the case of the antibacterial activity, a longer polypeptide of 31 amino acids (comprising the previously mentioned polypeptide) is required to recover the full activity. These results suggest that the mechanisms that are involved in coagulation and antibacterial effect are likely to depend on different parameters.

Table of content

Chapter 1

General introduction and objectives of the project

Drinking water is a problem of crucial importance in many areas of the world. Current water treatments have been criticized in terms of cost and safety.
My research subject concerns the expression and analysis of a protein from the seeds of *Moringa oleifera* tree, and their possible usage in alternative water treatment processes
In the following introduction I will present the multiple uses of *Moringa oleifera* tree, making emphasis in its seed extract application as a water-cleaning agent. In this context, the last part of the introduction will describe water cleaning processes as it is achieved currently in standardized water cleaning plants.

1.1 Introduction

What is *Moringa oleifera* ?

The *Moringaceae* is a family comprising 14 known species all of which belong to the genus *Moringaceae*. For example, *Moringa stenopetala* is native to Ethiopia and northern Kenya. *M. peregrina* is found in the Sudan, Egypt, the Arabian peninsula and as far north as the Dead Sea. *M. ovalifolia* grows in Angola and Namibia. However, the best known member of the genus, and the one that call our interest is *Moringa oleifera*, a fast-growing, drought-resistant tree native to sub-Himalayan tracts of northern India but now distributed worldwide in the tropics and sub-tropics (many countries in Africa, Arabia, South East Asia, the Pacific and Caribbean Islands and South America). Commonly known as the 'horseradish' tree (because of the taste of a condiment prepared from the roots), or 'drumstick' tree (because of the shape of the seed pods), *M. oleifera* has a host of other country specific vernacular names, an indication of the significance of the tree around the world. (Price, 1985; Parker et al. 2001)

Why *Moringa oleifera* ?

The *M. oleifera* tree is one of the most fully exploited tropical plants a according to the Educational Concerns for Hunger Organization (FI, USA) and the National Council of Churches (NY, USA). As it was mentioned, this plant it is now widespread and due to this, there are a wide variety of applications depending on the local necessities. The next paragraph will present an overview of *M. oleifera* tree applications. (Ramachandran et al., 1980 ; Booth and Wickens, 1988; Morton, 1991; Ram, 1994)
First, Moringa trees are well suited for being used in alley cropping systems because it has a rapid growth, long taproot, few lateral roots, minimal shade and large production of high-protein biomass. Furthermore incorporation of Moringa leaves into the soil before planting

can prevent damping off disease (*Pythium debaryanum*) among seedlings. In the same application leaves are employed as a domestic cleaning agent. For example crushed leaves are used in some parts of Nigeria to scrub cooking utensils or to clean walls. Since cattle, sheep, goats, pigs, and rabbits readily eat their leaves, it is employed for animal forage. Leaves can also be used as food for carp and other fish.

Regarding the applications directly related to human feeding it worth mentioning that *Moringa oleifera* leaves are an exceptionally nutritious source of vitamin A, B and C, as well as minerals, specially iron (it is prescribed for anemia in the Philippines). If the plant itself is young, it can be used as a tender vegetable (Ram, 1994; Saroj et al., 1995, 1996; Yabes-Almirante C and Lim C. H., 1996) .

The soft, spongy wood makes poor firewood, but the wood pulp is suitable for making newsprint and writing paper. Moreover from the wood it is extracted a blue dye, which was used in Jamaica and in Senegal. Also a gum produced from a cut tree trunk has been used in calico printing, in making medicines and as a bland-tasting condiment. In fact, medical applications are not rare (Ramachandran et al. 1980 ; Eilert et al, 1981). It can be said that every part of the tree is widely used to make a wide variety of traditional medicines. Other applications involve the production of ropes or mats because the tree can be beaten into fibers and tanning hides from the bark and gum.

Regarding pods, cooked pods reportedly taste like asparagus. Tinned pods are exported and they are eaten much like green beans. Furthermore, boiled flowers are commonly used for tea (in Haiti it is prescribed for colds) and as a source of honey because the tree is in flower during 8 months of the year. It has to be mentioned that other less classical applications are used. For example in many countries Moringa trees are planted in gardens and along avenues as ornamental trees and as live fencing around gardens (Price, 1985; Ghasi et al., 2000)

Seeds are in pods (which can be 120cm long, depending on the variety) and are commonly eaten as peas if boiled or fried when still green (to remove the alkaloid and saponin content). Fully mature, dried seeds are round or triangularly shaped and the kernel is surrounded by a lightly wooded shell with three papery wings (see figure 1 in this section). They have 40% oil with a quality equal of olive oil (73% oleic acid). Used mainly for cooking, it is also employed in perfume, lubrication, soaps and light. Since oil is the most important commercial derivates from seeds there is a traditional manner of extracting it. This process involves roasting, mashing and boiling the seeds. Since the oil floats on the surface after overnight sitting, the seedcake left over after the oil extraction can be used as a soil fertilizer or in the treatment of turbid water as well as to clarify sugar cane juice and honey, avoiding in this way to boil it. This way of water cleaning is traditionally used to clarify Nile river water, mainly in Sudan (Jahn, 1981, 1986; Price, 1985; Muyibi and Evison, 1995).

Moringa oleifera and water treatment

As previously mentioned, the usage of natural active compounds to clean water is an alternative manner of obtaining drinking water. Diverse communities in the world employ different animal or vegetal compounds with of course, several degrees of efficacy. One of the firsts records of how to employ natural compounds for this goal cames from sanskrit writings (India), dating from several centuries BC, and making reference to seeds of the tree *Strychnos potatorum* as a clarifier. More recently, peruvian texts from the 16th and 17th centuries detail the use by sailors of powdered, roasted grains of *Zea mays* as a means of settling impurities. Furthermore, in China, people in villages use denatured collagen from hides and bones as well as the sap of *Aloe vera* leaves. Moreover, chilean folklore texts from the 19th century refer to water clarification using the sap from the 'tuna' cactus (*Opuntia fiscus indica*). Since the above methods have widely different efficiencies, a more recent systematic studies have shown that among the different plant derived materials tested, *M. oleifera* seeds have been shown to be among the most effective as a primary coagulant for water treatment (Jahn, 2001)

Fig 1 *Moringa oleifera* tree (left). The seeds are in long pods (center). The round or triangularly shaped seeds have the kernel surrounded by a lightly wooded shell with three wings (right) -from Price, 1985-

In the specific case of Moringa, the powder from its crushed seed kernels works as a natural flocculant, binding to the solids in water and causing them to sink to the bottom. Since bacteria in water are generally attached to solid particles, treatment with Moringa powder can remove 90 to 99% of the bacterial load. However, boiling or adding chlorine or bleach to the water is needed to render it completely safe to drink. A general rule of thumb is to use the powder from one Moringa kernel for every two liters of water when the water is somewhat

turbid, and one kernel per liter when the water is very turbid (Jahn 1979, 1981, 1984, Tauscher, 1994)

The traditional use of the *M. oleifera* seeds for domestic household water treatment has been limited to certain rural areas in the Sudan. Village women collecting their water from the River Nile would place powdered seeds in a small cloth bag to which a thread is attached. This would then be swirled around in the turbid water. Water soluble proteins released from the powdered seeds, attach themselves to, and bind between, the suspended particles forming larger, agglomerated solids. These flocculated solids would then be allowed to settle prior to boiling and subsequent consumption of the water (Berger, 1984 ; Gupta and Chaudhuri, 1992 ; Folkard and Sutherland, 1996)

Molecular analysis of seeds

The majority of the early work (during the 90's) regarding the potential application of *Moringa oleifera* seeds extract was performed with a view to establishing the viability of using the seeds within household water treatment practices. Pilot plant trials and full scale trials done in Malawi, in collaboration with the Polytechnic of Malawi demonstrated that the seeds could be used effectively on continuous flow systems producing treated water quality similar to that obtained using aluminum sulphate (Jahn 1979, 1981, 1984 ; Berger 1984; Ndabigengesere, 1988; Gupta and Chaudhuri, 1992).

Analyses performed by Professor Tauscher at the University of Karlsruhe, Germany revealed that the coagulant properties of the seeds are due to the presence of low molecular weight cationic proteins having a molecular weight of 6.5 kDa and an isoelectric point above pH 10 (Tauscher, 1994; Gassenschmidt et al., 1995). Moreover, amino acid sequencing and analysis showed that these cationic polypeptides had a high content of glutamine, arginine and proline. However, the amino terminus was blocked by pyroglutamante rendering it impossible to identify the complete sequence. That protein, called MO2.1, exhibited an effect comparable to that of a synthetic cationic polymer

Challenges of water clearing standard treatments

An adequate supply of drinking water implies several basic parameters. Not only involves ensuring a continuous availability of water of good quality and maintenance of facilities and equipment but also the construction of institutional frameworks; applying management practices, appropriate technologies, and full-cost accounting. In developing countries, however, management of water supply and sanitation systems is often poor (see figure 2 in this section), resulting in interruptions in the provision of services and sometimes in the

complete collapse of systems. When the latter happens, users may be obligated to resort to traditional water sources, which may be contaminated. Contamination of distribution pipelines due to intermittent supply, low water pressure in the distribution network, inadequate wastewater collection systems and leaking pipes are also common problems in developing countries. If contaminated water penetrates distribution mains, water that has already been treated and disinfected may become recontaminated (World Health Organization, 2003).

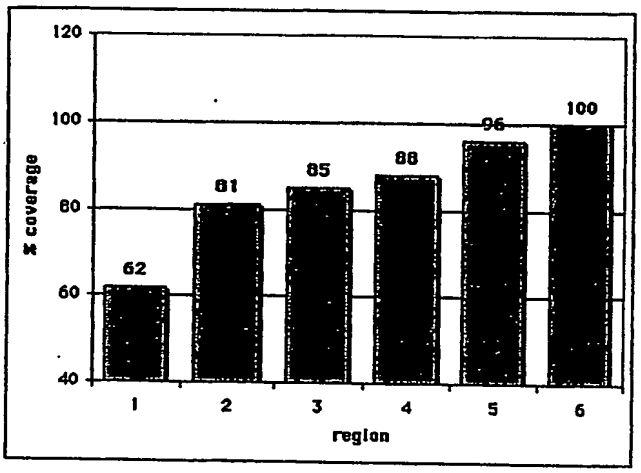

Fig 2 Regional water supply coverage in 2000 : 1:Africa, 2:Asia, 3:Latinoamerica, 4: Oceania, 5:Europe, 6:USA & Canada (from WHO, 2003)

Definition of raw water

Raw surface water contains particles in suspension as well as larger solid matter. On one hand, particles, which are greater than one micron in diameter, are considered silt, and settle out rapidly due to their relatively large size and density without the need to coagulate. However turbidity particles found in raw surface water can be up to 50 microns in diameter. On the other hand, smaller particles that range in size from 0.001 to one micron in diameter have a colloidal structure. Since the particles are stabilized by surface negative charges they repel each other. As these materials require days to months for complete settling, the rate of settling of these colloidal particles must be increased in the water treatment process (WHO, 2003)

In brief, water treatment usually comprises two main processes: clarification and disinfection. Both of them will be better described in the following paragraphs. At the end of this process water should taste good and look good — meaning neither odor nor coloration.

Water clarification: coagulation and flocculation

This first process, water clarification, is basically a two step process. The first one called coagulation is accomplished firstly through the addition of positively charged agents (aluminum sulphate, aluminum sodium, ferric chloride or ferric sulfate) that will neutralize and as a consequence, destabilize the colloidal structure formed by the tiny particles. After that moment, all these tiny particles do not repel each other anymore and are potentially able to agglomerate into larger, heavier. Next, water is stirred and then kept in a settling tank where the globs, or flocs, sink to the bottom. This second process (flocculation) involves the formation of "bridges" among the particles, accelerating in turn, the precipitation (see figure 3 in this section). The basic difference between coagulant and flocculant agents is the size. Meanwhile coagulants agents are positively charged molecules with a relatively low molecular size (50 to 150), flocculants agents can be cationic, anionic or non ionic and tend to be larger (100.000-1.000.000). For each sample, the appropriate coagulation/flocculation agent needs to be chosen empirically (NALCO, 1988)
Next, after the flocculation step, water is pumped very slowly across a large basin where much of the remaining floc and solid material accumulates at the bottom of the basin.

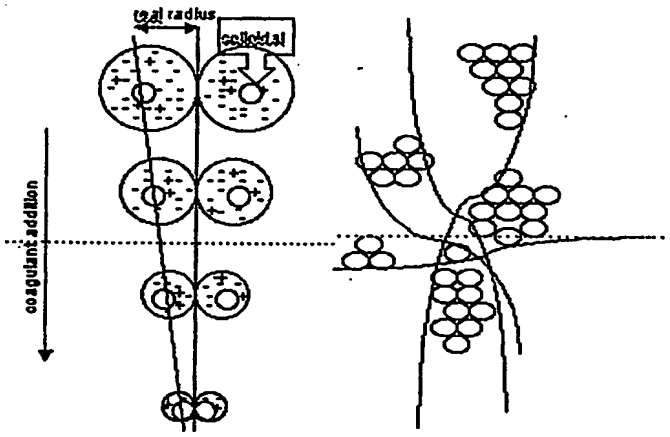

Figure 3 Water clarification. Coagulation (left): the coagulant agent addition neutralizes the charge of the particles decreasing their real radius. During flocculation (right), the neutralized particles are assembled to accelerate the sedimentation (from NALCO, 1988)

Water disinfection: filtration, chlorination, ozone oxidation and ultraviolet light

The final step involves disinfection of the clarified water through two approaches : filtration and chemical disinfection.

Filtration is a nonspecific disinfection method because it involves passing the water through layers of granular material to remove microorganisms –including viruses, bacteria and protozoans such as Cryptosporidium– and any remaining floc and silt. This stage of purification mimics the natural filtration of water as it moves through the ground. (NALCO, 1988)

After the water is filtered, it is treated with chemical disinfectants to kill any organisms that might have remained through the filtration process. The most widely used approach is disinfection using chlorine. When chlorine combines with organic material producing potentially dangerous trihalomethanes (THMs). Another physico-chemical treatment worth mentioning is ozone oxidation and ultraviolet light irradiation. Both of them are effective disinfectant processes, but unlike chlorine, they do not stay in the water after it leaves the treatment plant, so it offers no protection from bacteria that might be in the water pipes.

Despite that the coagulation properties of *Moringa oleifera* seed extract were already described, the mechanism of action is poorly understood.

1.2 Aim

In the next chapter it will be described the production and characterization of one protein from *Moringa oleifera* seeds. This 6.5 kDa cationic polypeptide was previously identified from a flocculation fraction in the seeds. Its primary sequence was determined only partially by Edman degradation because the N-terminus was blocked by pyroglutamate. From the primary sequence a gene was synthesized and cloned in an expression vector. In that opportunity they could not obtain any expression product, thus reducing in that way the feasibility of this protein as a biotechnological product by molecular cloning (Tauscher,B., 1994 ; Gassenschmidt et al., 95).

My goal is to express this polypeptide in bacteria as a transitory fusion protein, in order to have pure enough material. Once this goal is achieved, its efficiency as a water cleaning agent, measured through sedimentation of colloidal particles and bacteria, will be compared to that of the whole *Moringa oleifera* seed extract.

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Chapter 2

Expression of a Plant-Derived Peptide Harboring Water-Cleaning and Antimicrobial Activities

Results published as : M. Suarez , J. M. Entenza, C. Doerries, E. Meyer, L. Bourquin, J. Sutherland, I. Marison, P. Moreillon, N. Mermod. Biotechnology and Bioengineering 81 (1) : 13-20 (2003)

Results corresponding to Figure 1 to 4 result directly from my own work.
E. Meier is the responsible of the cloning strategy and JM. Entenza and L. Bourquin of Table I results.

ACCELERATED PUBLICATION

Expression of a Plant-Derived Peptide Harboring Water-Cleaning and Antimicrobial Activities

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Abstract: Drinking water is currently a scarce world resource, the preparation of which requires complex treatments that include clarification of suspended particles and disinfection. Seed extracts of *Moringa oleifera* Lam., a tropical tree, have been proposed as an environment-friendly alternative, due to their traditional use for the clarification of drinking water. However, the precise nature of the active components of the extract and whether they may be produced in recombinant form are unknown. Here we show that recombinant or synthetic forms of a cationic seed polypeptide mediate efficient sedimentation of suspended mineral particles and bacteria. Unexpectedly, the polypeptide was also found to possesses a bactericidal activity capable of disinfecting heavily contaminated water. Furthermore, the polypeptide has been shown to efficiently kill several pathogenic bacteria, including antibiotic-resistant isolates of *Staphylococcus, Streptococcus,* and *Legionella* species. Thus, this polypeptide displays the unprecedented feature of combining water purification and disinfectant properties. Identification of an active principle derived from the seed extracts points to a range of potential for drinking water treatment or skin and mucosal disinfection in clinical settings. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* 81: 13–20, 2003.

Keywords: coagulation; antimicrobial peptide; drinking water; disinfection

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INTRODUCTION

The treatment of water to render it fit for human consumption has become a problem of central importance, both in developing and in developed countries. In developing areas, the quality of drinking water is often insufficient and hazardous to health. In developed countries, water purification processes use chemicals, despite the fact that their safety for health during long-term use and impact on the environment remain under question. As a result, it is desirable to find sustainable alternatives more friendly to human health and to the environment.

Water treatment usually comprises water clarification and disinfection. The turbidity of water often results from the presence of negatively charged particles in a colloidal structure, the clarification of which requires acceleration of the sedimentation rate. For this purpose, positively charged agents are used to neutralize the negative charges of the colloid, in a process called coagulation. In developed countries, salts of aluminum and other metals are often used (Boisvert et al., 1997; Nalm et al., 1998; van Benchosten and Edzwald, 1990), despite the concern that they may induce Alzheimer's or other disease (Crapper et al., 1973; Martyn et al., 1989; Miller et al., 1984). Although synthetic organic polymers are used increasingly for water treatment in conjunction with metal salts, efficient substitutes for the latter are not available at present (Odegaard, 1998; Parker et al., 2001).

Water disinfection also often makes use of chemical additives, with chlorine and chloramine being two of the most widely used ones. While the benefits of water disinfection are clear, concerns have also been raised over safety issues concerning disinfectants. For example, disinfectants and their by-products may be associated with increased risks of

cardiovascular diseases, cancers, and birth defects. Although such risks are low, associations with such diseases could not be ruled out unambiguously (Arbuckle et al., 2002; Bove et al., 2002; Woo et al., 2002).

Naturally occurring alternatives to currently used coagulants and disinfectants have also been considered, including ones found in cultivated plants. Of particular interest are the seeds of a tropical tree, *Moringa oleifera* Lam., as they contain an active coagulating compound traditionally used for the clarification of drinking water in rural areas of Sudan and Malawi (Eilert et al., 1981; Okuda et al., 2000). The coagulating activity of *Moringa* sp. seeds is still a mater of debate, as it has been ascribed either to low molecular mass cationic polypeptides (Tauscher, 1994) or to a non-proteinaceous compound of unknown structure (Okuda et al., 2000). In addition, such seed extracts are capable of bacterial aggregation and removal, with efficacy similar to that of aluminum salts and other commonly used water treatment agents (see Madsen et al., 1987, and references therein). These properties suggest that seed extracts may be promising alternatives to the currently used chemical water treatment agents.

In this paper we describe the expression and characterization of one of the *Moringa* sp. seed polypeptides. Our results show that is acts as a water clarification agent that coagulates particles and bacteria in suspension. In addition, we show that it also possesses an antibiotic activity that leads to growth inhibition and killing of bacteria, including antibiotic-resistant human pathogens. Thus, *Moringa* seed polypeptides might represent environment-friendly substitutes to commonly used coagulation and disinfecting agents.

MATERIALS AND METHODS

Plasmids

A DNA sequence was designed to encode the MO2.1 polypeptide sequence of Gassenschmidt et al. (1995) (see Fig. 1A). Recombinant or synthetic forms of this polypeptide were termed Flo in this article. The double-stranded oligonucleotide was synthesized using a PCR assembly strategy, as described previously (Horton et al., 1989). The oligonucleotide sequence was designed so that its codons are optimized for *Escherichia coli* expression and so that SapI and PstI restriction sites are located at its extremities. The pTYB11 plasmid of the IMPACT expression system (intein-mediated purification with an affinity chitin-binding tag system, New England Biolabs, Inc., Beverly, MA) was selected for cloning and expressing the *Moringa* seed Flo protein in *E. coli*. The oligonucleotide was ligated to SapI/PstI-digested pTYB11 vector so that the sequences encoding the N-terminus of the target protein Flo, an internal protein self-cleavage site (intein), and chitin-binding domain are fused. Positive clones were verified by sequencing.

Protein Expression and Purification

Flo protein was expressed using the pTYB vectors and *E. coli* ER2566 strain (New England Biolabs). To induce expression of the fusion protein, 0.3 m*M* IPTG was added to an exponentially growing culture at an A_{600} of 0.5–0.6 during 2 h at 27°C. The bacterial culture, extract preparation, and purification conditions were as recommended by the manufacturer (New England Biolabs). In brief, a 1.5-liter bacteria culture volume (A_{600} = 0.5–0.6) was centrifuged, and cells were lysed by sonication. Extracts were clarified by centrifugation and loaded onto an equilibrated chitin bead column. After being washed, the column was filled with 50 m*M* DTT containing buffer, which was incubated in the column for 40 h at room temperature to allow for self-cleavage of the Intein-containing fusion peptide. Flo was eluted, and its presence was confirmed by gel electrophoresis. Finally, precursor protein was eluted with stripping buffer, and the column was recycled.

Total cell protein extracts were analyzed using 10% SDS-PAGE (Laemmli, 1970). For protein quantification, gels were stained using cypro-orange and analyzed using scanning software (Storm 840, Molecular Dynamics, Sunnyvale, USA). The Flo polypeptide was analyzed by Tris-tricine SDS-PAGE (Schagger and von Jagow, 1987). For gel fixing and staining, a protocol suitable for small basic proteins was followed (Steck et al., 1980).

Synthetic Flo was synthesized at the Peptide–Protein Chemistry Facility of the University of Lausanne using the amino acid sequence of *Moringa oleifera* Lam. seed component M.O. 2.1 (Gassenschmidt et al., 1995). *Moringa* sp. seed extract was obtained as a commercial preparation (Phytofloc, Optima Environment Inc., Nyon, Switzerland). Briefly, a ground presscake of *Moringa* sp. seeds was mixed with saltwater at 1:5 w/v ratio. The extract was filtered and heated at 80°C. Precipitated solids were removed by centrifugation and the clarified liquor was concentrated by filtration through 5-kDa cut-off membranes. Further inquiries on the use of Phytofloc and Flo (patent pending) may be directed to Optima Environment SA.

Coagulation Assays

Coagulation activities were evaluated using a 1 mg/mL suspension of 3.5-7 μm diameter glass particles (Spheriglass 5000, Potters-Ballotini, U.K.) in 2 mL of 50 m*M* phosphate buffer, pH 7.0, to mimic turbid water. Stirring was kept continuously at 800 rpm, and OD was recorded at 500 nm (LabVIEW software/National Instruments Corporation) in a spectrophotometer. After 5 min of continuous stirring, the compound to be tested was added to a final concentration of 10 μg/mL, unless otherwise noted, and stirring was continued for 15 min. The flocculation efficiency was estimated from a linear regression performed on time points corresponding to 4 min before the addition of the flocculating preparation (basal sedimentation) and 4 min after the addition of the flocculating preparation (coagulation-mediated sedimentation).

Antibacterial Effects

Exponentially growing *E. coli* ER2566 was centrifuged and suspended in a same volume of 10 m*M* phosphate, pH 7.0,

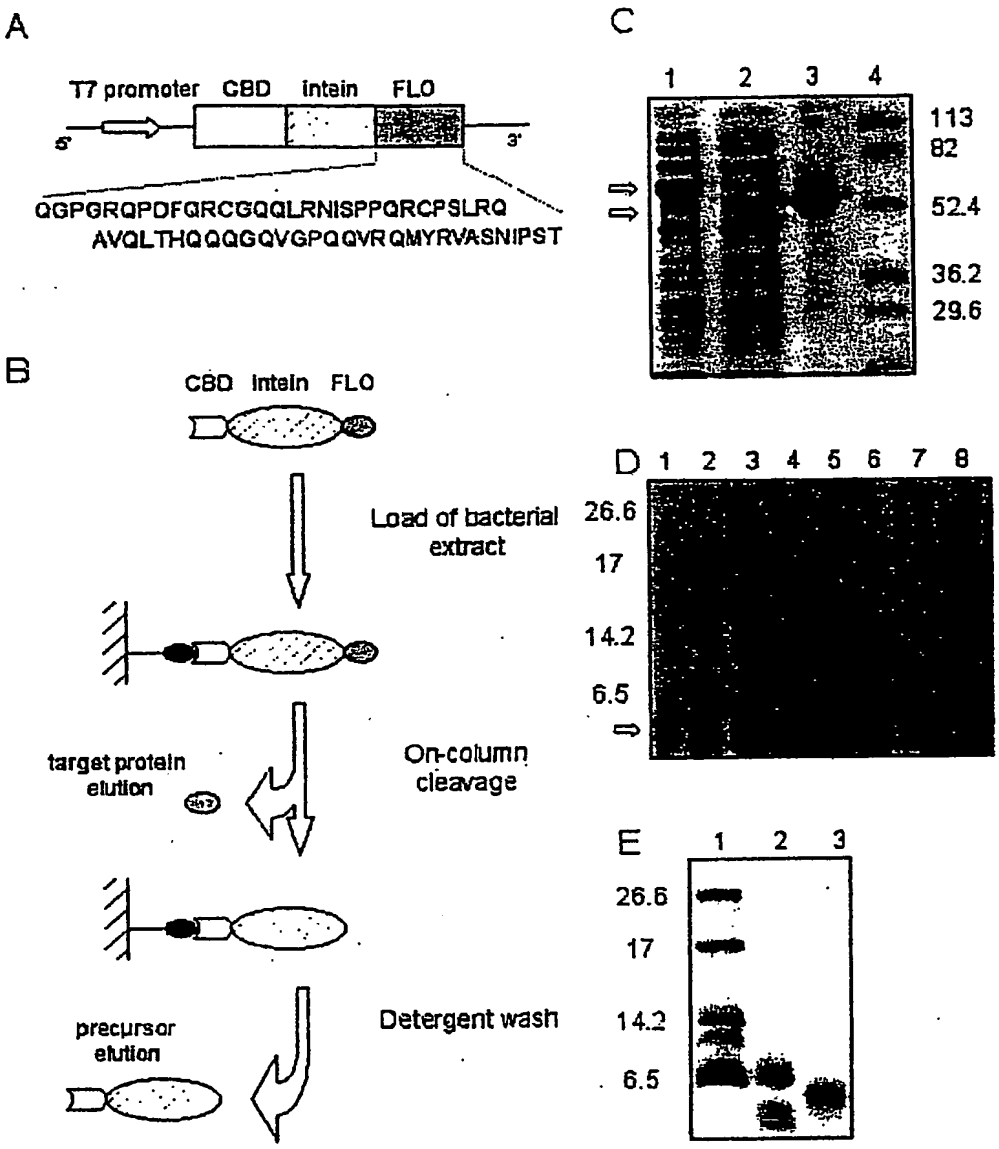

Figure 1. Flo expression and purification. (A) Structure of the Flo fusion protein expression vector. The Flo coding sequence (shaded box) was inserted downstream of sequences encoding the self-cleavage intein protein domain (striped box) fused to the chitin-binding domain (CBD, doted box), under the control of a regulated T7 phage promoter. Sequence of the Flo polypeptide, as released from the intein sequence after self-cleavage, is shown below. (B) Scheme of the purification process. The bacterial extract containing the fusion protein was loaded onto a chitin-linked (closed ellipse) bead column, where the fusion protein is retained through its chitin-binding domain. The column was then incubated with thiols, which results in a specific self-cleavage of the intein, releasing the Flo polypeptide. The remainder of the fusion protein is then eluted in detergent buffer to recycle the column. (C) SDS-PAGE analysis of bacterial extracts. Equivalent fractions of the purification intermediates were loaded as follows: crude extract from IPTG induced cells (lane 1); chitin column flow through (lane 2); eluate of remaining part of the fusion protein after self-cleavage (lane 3); protein molecular weight marker (lane 4); At the left, the upper arrow indicates the fusion protein (61.5 kDa) and the lower arrow indicates the fusion protein after cleavage and elution of Flo (55 kDa). (D) Tris-tricine SDS-PAGE analysis of Flo eluate fractions. Lanes 1 and 2: 1 and 2 μg of chemically synthesized Flo were loaded, respectively. Lanes 3–8: sequential fractions of Flo elution. At the right, the position of markers is indicated in kDa. The arrow indicates the position of Flo. Trace amount of polypeptides whose migration corresponds to multimers of Flo was occasionally noted in highly concentrated fractions (lanes 2–6). (E) Tris-tricine SDS-PAGE analysis of synthetic Flo and seed extract; 2.5 μg of either total protein seed extract (lane 2) or synthetic Flo (lane 3) was resolved in parallel. Masses of molecular weight markers (lane 1) are as indicated on the left.

buffer. Alternatively, incoming water was collected at a waste-water treatment facility. Phytofloc, synthetic Flo, or BSA was added and incubated with bacteria as specified in the figure legends at 37°C. Bacteriostatic effects were measured by adding LB growth medium to the bacterial suspension to obtain $A_{600} = 0.1$, the cultures were incubated at 37°C under agitation, and the culture growth was followed by A_{600} measurements. Alternatively, particles and viable cells were counted either directly after the incubation or after additional washes of the cells in phosphate buffer to remove flocculating proteins. Assays of particle size and number were performed with a CASY cell counter (Schärfe System Inc., Reutlingen, Germany), and cell viability was assessed by plating bacterial suspensions on non-selective LB medium dishes. Standard deviations of independent measurements ranged from 2% to 20% of the mean.

Determination of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentrations (MBC)

Methicillin-resistant *Staphylococcus aureus* P8 (Entenza et al., 2001), *Streptococcus mitis* (Entenza et al., 1999), *Streptococcus pneumoniae* (Moreillon et al., 1990), *Enterococcus faecalis* (clinical isolate, Lausanne University Hospital), *Legionella pneumophila* serotype 1 (nosocomial isolate, Ticino Bacterioserological Institute, Lugano, Switzerland), *Streptococcus pyogenes* ATCC 19615, and *E. coli* ATCC 25922 (NCCLS strain collection) were grown at 37°C without aeration, in either Mueller Hinton broth (MHB), buffered yeast extract medium (BYEα), or buffered charcoal yeast extract (BCEYα; Difco Laboratories, Detroit, MI) or on Columbia agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD) supplemented with 4% blood. Approximately 5×10^5 CFU/mL of bacteria were suspended in phosphate buffer as above and incubated at 37°C with indicated amounts of Flo or *Moringa* sp. seed extracts. After 24 h of incubation (48 h for *L. pneumophila*), 0.01- and 0.1-mL volumes of bacterial suspension were spread on nutrient agar, and the plates were incubated for an additional 24 h (48 h for *L. pneumophila*) at 37°C before colony counts. The MIC was defined as the lowest concentration of Phytofloc or Flo inhibiting bacterial growth. The MBC was defined as the lowest drug concentration resulting in a ≥99.9% decrease in viable counts as compared to the original inoculum (National Committee for Clinical Laboratory Standards, 2000). Phase contrast microscopy indicated that the decrease in bacterial viability was not due to aggregation.

RESULTS

Previous work on the coagulating activity associated with *M. oleifera* Lam. seed extracts had indicated that the activity co-purifies with low molecular weight proteins. The sequence of one of these proteins was determined and shown to be a positively charged 6-kDa polypeptide (Tauscher, 1994). However, previous attempts to express a recombinant form of this protein and to demonstrate an associated coagulation activity were not successful. In the present study, we have addressed this issue using either a synthetic form of this polypeptide or with a bacterially produced recombinant protein.

Protein Cloning, Expression, and Purification

Using the previously determined protein sequence, we reconstructed a synthetic gene optimal for the expression in *E. coli* of the recombinant *M. oleifera* Lam. seed protein, which we termed Flo. Previous work had demonstrated that the expression of positively charged peptides may be toxic to *E. coli* (Piers et al., 1993). Therefore, the expression vector was designed such that the highly positively charged Flo is expressed as a fusion polypeptide with the negatively charged intein and chitin-binding domains (Fig. 1A). The chitin-binding domain allows for convenient purification of the fusion protein, while intein allows post-translational cleavage of the precursor protein, as described previously (Perler, 2000) (Fig. 1B). Over-expression of a fusion protein of the expected size was obtained (Fig. 1C), yielding approximately 30% of the total bacterial protein content. Purification and autocatalytic cleavage of the fusion protein allowed the recovery of approximately 1 mg of purified Flo per liter of bacterial culture (Fig. 1D).

Analysis of Flo amino acid sequence indicated significant similarity with the 8 kDa heavy chains of the napins and mabinlins, except that the N-terminal portion of the polypeptide is missing in Flo (M. Suarez and N. Mermod, unpublished results). The napins and mabinlins belong to the plant 2S albumin protein family, the most abundant storage proteins in plant seeds (Broekaert et al., 1997). Thus, Flo is expected to represent a truncated version of its *M. oleifera* Lam. seed 2S protein counterpart. As expected from this finding, bacterial or synthetic Flo migrated just below the heavy chain of the seed extract major protein (Fig. 1E).

Coagulation Activity of Flo

The coagulation properties of chemicals and protein extracts are usually assessed using suspensions of particles, such as glass microbeads, that mimic the negatively charged properties of particles found in natural turbid waters (Tauscher, 1994). Flo and the seed extract were therefore added to continuously agitated suspension of glass microbeads, and sedimentation was estimated by following the decrease in optical density. Little sedimentation occurred before or after the addition of buffer (Fig. 2A). However, efficient coagulation occurred with saturating amounts of seed extract (Fig. 2B) and with chemically synthesized or bacterially produced Flo (Fig. 2C,D). Use of subsaturating amounts of the synthetic peptide and of the seed extract indicated comparable activities in this semiquantitative assay (Fig. 2E,F), which correlates well with the similar amounts of the major polypeptides observed in the two preparations (Fig. 1E).

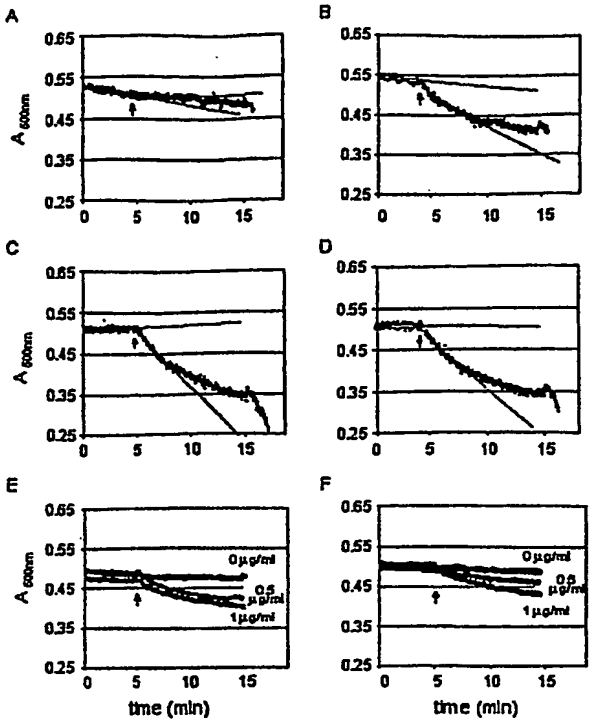

Figure 2. Coagulation activity of Flo. The glass particle suspension sedimentation assay was performed in spectrophotometer cells as described in Materials and Methods, and optical density measurements at 600 nm were taken at 1-s intervals. After 5 min of stirring, seed extract (B), synthetic Flo (C), or bacterially expressed and purified Flo (D), respectively, were added to a final concentration of 10 µg/mL, as indicated by the arrow. In panel A, a similar amount of buffer only was added. The slopes of the sedimentation curves, before and after addition of the compound to be tested, were estimated by linear regression calculations as described in the Experimental protocol, and are shown as straight lines. (E, F) Titration experiments performed with the indicated subsaturating amounts of the seed extract and synthetic Flo, respectively.

Antibacterial Effects of Flo

Moringa sp. seed extracts were shown previously to flocculate bacteria and to possess antimicrobial activity (Eilert et al., 1981; Madsen et al., 1987). However, the agent responsible for the flocculation activity was not identified, while the antimicrobial activity was ascribed to plant-synthesized derivatives of benzyl isothiocyanates, a known antibacterial compound. Nevertheless, we set up to evaluate the potential effects of Flo on *E. coli*. Exponentially growing bacteria were incubated with various concentrations of Flo and returned to culture conditions to monitor growth. Incubation with 2 mg/mL of either seed extract or Flo resulted in strong inhibition of bacterial growth (Fig. 3A). Growth inhibition was detectable at lower Flo concentrations, with an IC_{50} of approximately 100 µg/mL (Fig. 3B). Incubation with bovine serum albumin, used as a negative control, indicated that the antibacterial effect is specific to Flo.

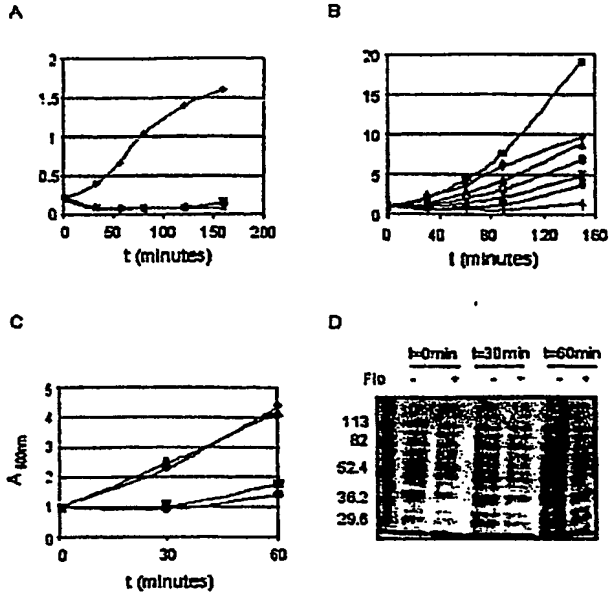

Figure 3. Effect of Flo on *E. coli* culture growth. (A) Effect of the *Moringa* sp. seed extract and of bacterial Flo protein. An exponential-phase *E. coli* culture was centrifuged and incubated for 2 h at 37°C in phosphate buffer alone (filled diamonds) or in phosphate buffer supplemented with seed extract (filled triangles) or with synthetic Flo (filled squares) at a final concentration of 2 mg/mL. Bacteria were then diluted to A_{600} = 0.1 in LB growth medium and incubated at 37°C under agitation. Optical density measurements were then recorded as indicated at 600 nm. (B) Exponentially growing *E. coli* culture was processed as indicated for panel A except that bacteria were incubated with different concentrations of synthetic Flo (in mg/mL): 0 (filled squares), 0.1 (filled triangles), 0.25 (open circles), 0.5 (stars), 1 (filled circles), or 2 (plus signs). BSA at 2 mg/mL was used as a control (filled diamonds). (C) Assay for bacterial resistance to the antibiotic effect of Flo. *E. coli* culture consisted either of a fresh culture of bacteria (untreated bacteria) or of a culture previously incubated in presence of peptide in two successive rounds, as in panel A, where the bacteria that grew eventually were collected. These cells were incubated for a third cycle with either buffer (filled triangles) or with 2 mg/mL Flo (filled squares). Untreated cells were incubated in parallel either with buffer (0 mg/mL Flo, filled diamonds) or with Flo (2 mg/mL, filled circles). (D) Protein synthesis by bacteria incubated or not with synthetic Flo. Similar volumes of the culture of treated cells shown in panel C, either incubated with buffer (minus signs) or with 2 mg/mL of Flo (plus signs), were collected at the indicated time, bacteria were precipitated and total cell proteins were separated by SDS-PAGE and stained with Coomassie blue.

The inhibition of *E. coli* growth was found to be transitory, with resumption of growth after 3–6 h (data not shown). This suggested that some bacteria present in the culture may be spontaneously resistant to a bactericidal activity of Flo, or alternatively that the culture may eventually escape the bacteriostatic effect of Flo, for instance, after degradation of the polypeptide. In order to select for putative resistant bacteria, Flo was added to growing cells to achieve growth inhibition, and, after resumption of growth, cells were collected and incubated again with Flo. Bacteria that had grown were challenged for a third time with the polypeptide, and Flo inhibited cell growth again in the same

way as observed with untreated cells (Fig. 3C). Thus, the ability of the culture to escape the effect of Flo is unlikely to be due to a minor proportion of previously resistant bacteria. To ascertain that the decrease in absorbance of the culture after the addition of Flo is due to growth inhibition rather than to the aggregation or sedimentation of the bacteria, total cell extract proteins were resolved by SDS-PAGE. This showed that bacteria treated with Flo did not synthesize proteins, in contrast to control cells (Fig. 3D). Therefore, Flo blocks *E. coli* biomass accumulation and prevents cell growth.

Visual inspection of Flo-incubated *E. coli* revealed that the peptide aggregated the bacteria, as indicated by the appearance of defined particles or flocs (data not shown). Analysis of the size and number of bacterial particles with a cell counter revealed that Flo causes an increase in the partitioning of cells in particles greater than 2 μm, indicative of cell flocculation (Fig. 4A). Thus 6 mg/mL of Flo led to flocculation of 50% of the cells. Cell aggregation was accompanied by a decrease in the count for viable cells when spread over solid growth medium, which might conceivably result from cell death and/or from cell aggregation.

To determine if Flo has a bactericidal activity, flocs were resolved by several cycles of washing to remove Flo, and the cell size and number were determined (Fig. 4B). With up to 2 mg/mL of Flo, cells were recovered with a size profile consistent with complete resolution of the flocs. However, treatment with Flo significantly decreased the number of recovered cells, with an IC_{50} value of approximately 1 mg/mL. Flo at 6 mg/mL and above decreased particle counts to background values of 10 particles/mL and lowered viable cell count by 3 orders of magnitude, indicating that cell lysis had occurred, while approximately 99% of the remaining particles were non-viable (Fig. 4C,D). This clearly shows that Flo has a bactericidal activity.

To address whether Flo might be used to disinfect contaminated water, samples from the input of a waste-water treatment facility were tested similarly. Again, concentrations of 1–6 mg/mL of Flo were found to decrease viable cell counts by several orders of magnitude (Fig. 4D), implying a bactericidal activity on a range of bacteria and conditions.

To further assess Flo antimicrobial specificity, several Gram-positive and -negative bacteria were tested using protocols standardized for the assay of antibiotic compounds (National Committee for Clinical Laboratory Standards, 2000). A bacteriostatic effect was observed against several human pathogens such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *L. pneumophila*, and minimal inhibitory concentration (MIC) values ranged between 0.8 and 5 mg/mL of Flo between and 5 and 20 mg/mL for the seed extract (Table I). Bactericidal activities (minimal bactericidal concentration, MBC) were also observed against these bacteria. Interestingly, such organisms are among the most problematic human pathogens resistant to commonly used antibiotics (Entenza et al., 2001). In contrast, Flo antibacterial activity was relatively lower on several other bacteria, including *E. coli*. This latter result is consistent with our previous finding that moderate concentrations of Flo mediate transitory bacteriostatic effects of *E. coli* which would not be detected over the 24 h time scale of the MIC and MBC assays. Thus, Flo displays selective antibacterial effects on a range of Gram-positive and Gram-negative human pathogens.

DISCUSSION

The purpose of this study was to identify active components in *Moringa* sp. seed extracts and to determine if recombinant derivatives such as the Flo polypeptide might have a coagulation and/or flocculation activity. Our results show that high yields of Flo can be obtained from *E. coli*. The coagulation test results showed a very efficient coagulation activity of the synthetic and bacterially produced Flo polypeptide. This effect was observed using two models for water clarification: the coagulation of glass microparticles and the flocculation of bacteria. These findings indicate that the Flo polypeptide possesses hallmark characteristics of efficient water clarification.

Inspection of the Flo sequence indicated that it is extremely rich in positive charges. This is reminiscent of the so-called peptide antibiotics found in animal and plants that display a bacteriostatic or bactericidal activity (Zasloff, 2002). Furthermore, Flo displays significant homology to part of the Napins and Mabinlins 2S albumin seed proteins, some of which possess antimicrobial activities (Broekaert et al., 1997). Therefore, we tested a possible antibiotic activity of Flo and found that it displays bacteriostatic and bactericidal activities on several Gram-positive and Gram-negative bacteria. *Moringa* sp. seeds have been shown to contain one or several antibiotic principles against a wide range of Gram-positive and -negative bacteria and against fungi. This antibiotic activity was previously attributed to plant-produced benzyl isothiocyanate derivatives (Eilert et al., 1981). Our study shows that at least part of the antibiotic activity of *Moringa* sp. seed extracts may stem from Flo-like polypeptides.

Antimicrobial peptides have attracted increasing attention recently because they can efficiently kill fungi and bacteria that are otherwise resistant to many commonly used antibiotics. They act by forming channels in bacterial membranes or by inhibiting essential enzymes, leading to cell death (Zasloff, 2002). The antibacterial effect of Flo might conceivably result from similar activities or from its bacterial flocculation effect. The latter possibility is unlikely, as the bacteriostatic action of Flo is exerted at lower concentrations than its flocculation effect. For instance, Flo concentrations required to otbain half-maximal effects on *E. coli* are 0.1 mg/mL for the bacteriostatic action, 1 mg/mL for the bactericidal activity, and around 6 mg/mL for cell flocculation. Furthermore, Flo causes cell death, which is not commonly associated with cell flocculation by chemical agents.

Results presented here suggest multiple uses for Flo-like polypeptides. *Moringa* sp. seed polypeptides may be valu-

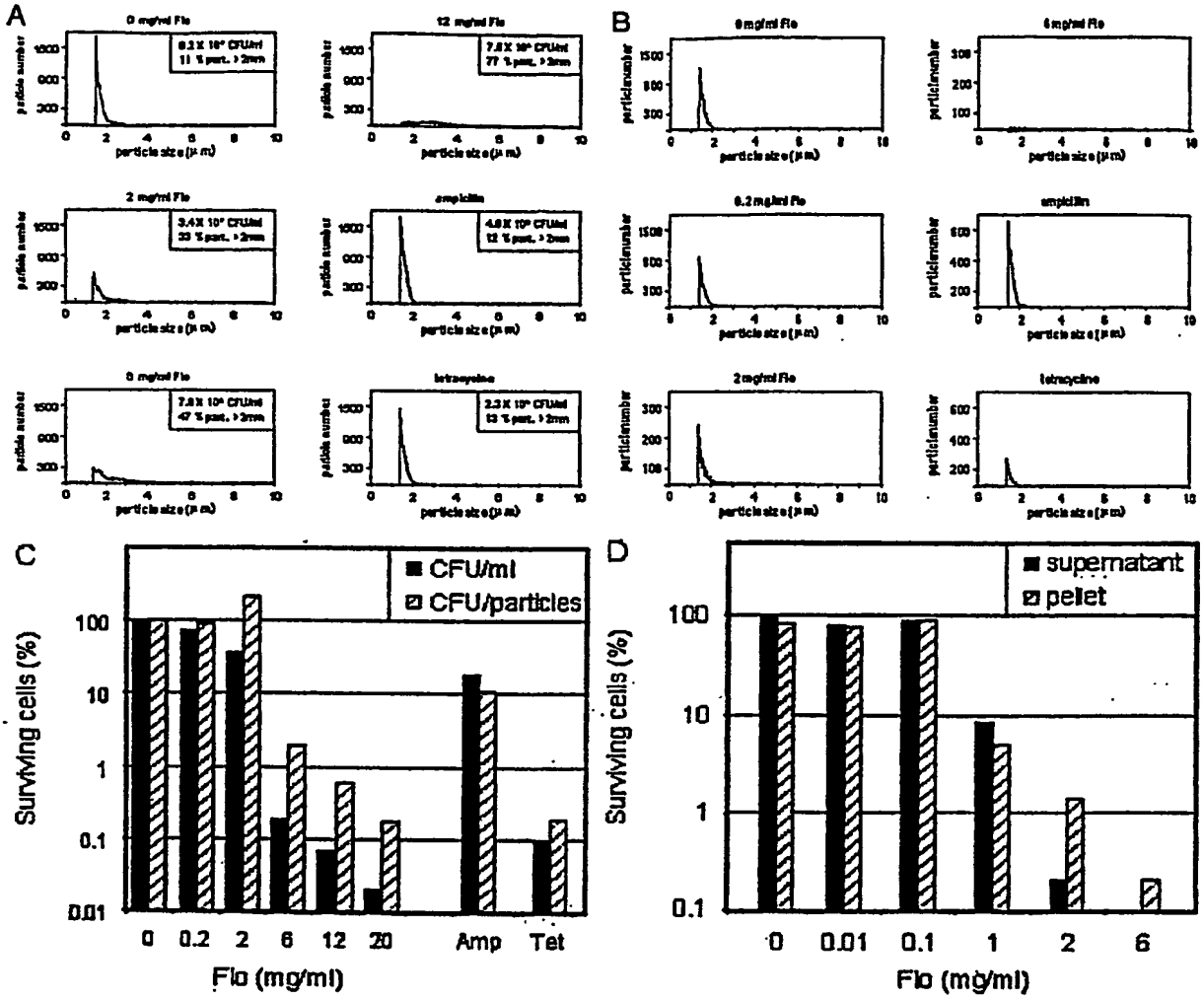

Figure 4. Effect of Flo on bacterial cell flocculation and viability. (A) Effect of the synthetic Flo protein on the cell particle size and number. An exponential-phase *E. coli* culture was centrifuged and incubated for 2 h at 37°C in phosphate buffer alone or in phosphate buffer supplemented by synthetic Flo at the indicated final concentration, or with 0.1 mg/mL ampicillin or 0.01 mg/mL tetracycline. Cells were then analyzed in a cell counter, and particle number is represented as a function of size. The inset shows the number of colony-forming units (CFU)/mL, scored after the suspension was spread on non-selective solid media, and the proportion of particles with a size above 2 μm, expressed as the percentage of the total number of counted particles. (B) Recovery of cells after incubation with Flo and Flo removal. Cells were incubated as described for A and then subjected to multiple wash cycles with phosphate buffer to remove Flo, as described in the Experimental protocol section. Cells were then analyzed in the cell counter as in A. (C) Effect of Flo on *E. coli* cell viability. Cell suspensions, incubated and washed as described in B, were spread on non-selective solid media, as in A. The cell viability is expressed as colony-forming unit values divided either by the volume of the original cell culture or by the total number of particles determined in B. Values are normalized to those obtained for cells incubated in phosphate buffer only (0 mg/mL), which was assigned a value of 100%, and ranged from 1×10^9 to 5×10^9 CFU/mL. (D) Effect of Flo on bacterial cell viability. Heavily contaminated waters from the input of a waste-water treatment facility were mixed with the indicated amount of Flo and incubated for 10 min, before spreading supernatant or sediment fractions on nonselective solid media as in A and incubating at 30°C for 24 h. Cell viability is expressed as colony-forming units, and values are normalized to those obtained for cells supplemented with phosphate buffer only (0 mg/mL), which were assigned a value of 100%. Initial bacterial loads ranged from 1×10^5 to 7×10^5 CFU/mL.

able alternatives to chemicals commonly used as food preservatives or for water disinfection and clarification. These polypeptides are unlikely to have toxic effects, as the seeds are currently used for the traditional treatment of drinking water and for the preparation of oil and various foods. Another advantage of treating water with such polypeptides is their biodegradability, unlike aluminum salts, for example, which remain as contaminants of treated waters and of the sediments. Finally, Flo and *Moringa* sp. seed proteins exert selective bactericidal effects and may clear water from several water-borne human pathogens. Clearing water pipes feeding both drinking water and centralized air conditioning

Table I. Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of Flo on test organisms (in mg/mL).

Bacteria	Seed extract		Flo	
	MIC	MBC	MIC	MBC
Staphylococcus aureus	9–18	12–32	2–5	5–10
Streptococcus pyogenes	12	12–24	2–5	2–5
Streptococcus mitis	50	>50	10	10
Streptococcus pneumoniae	16–32	16–32	1	2.5
Enterococcus faecalis	>50	>50	10	>10
E. coli	50	>50	10	>10
L. pneumophila	5	5	0.8	2–3

systems is an everlasting issue in modern infrastructures. The properties of Flo, and the fact that *Moringa* seeds can be obtained and processed at a large scale (Y. Poirier, personal communication), indicate that *Moringa* sp. proteins may be a viable alternative for such applications.

The finding that recombinant Flo has antibacterial activities indicates further potential biomedical applications. Flo was shown to kill several human pathogens, such as *Staphylococcus*, *Streptococcus*, and *Legionella* species, including strains resistant to commonly used antibiotics. Decolonization of patients carrying multiresistant staphylococci and streptococci by topical application has proved critical in interfering with the spread of resistant bacteria. Selectively targeting the pathogens while sparing the commensals is key to such interventions. Because of their selectivity for several human pathogens that colonize mucosal surfaces, Flo-like polypeptides might fit such applications.

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Chapter 3

Molecular analysis of the flocculation and antibacterial activity of Flo

In the previous chapter we have shown that both the synthetic and bacterially produced Flo polypeptides have an efficient coagulation activity (Suarez et al, 2002). This effect was observed using two models for water clarification: the coagulation of glass microparticles and the flocculation of bacteria. Surprisingly, we also identified in Flo an efficient antimicrobial activity.

Flo displays a highly cationic structure that is reminiscent of what is described for a wide group of molecules called antimicrobial polypeptides (AMPs). Many models are proposed to explain the manner that these AMPs exert their effect. What is known at present will be summarized below.

3.1 Introduction

Cationic antimicrobial polypeptides

Cationic polypeptides are important components of the innate defenses of all species (from insects to higher eukariotes including plants, invertebrates and vertebrates). Most of them kill microorganisms by forming pores in the cell membrane through interaction with the negatively charged phospholipids. Since cholesterol is a major mammalian cell membrane component, these peptides are not toxic for mammalian cells. (see fig 1 in this section)

Plants have only innate immune system, in which either constitutively or upon perception of microbial signals, chemical substances are produced to control microbial growth on their surfaces. The plant microbial peptides comprise thionins (with 6 or 8 cysteines), plant defensins, or two other peptides known as hevein-type and knottin-type. All of them posses antibacterial and antifungal activity (Schröder, 1999).

Some of the cationic polypeptides are more specific against bacteria while others are mainly antifungal (Broekaert et al, 1997; Hancock and Lehrer, 1998 ; Schröder 1999; Zasloff, 2002)

Therefore, we tested a possible antibiotic activity of Flo. Although Flo does not display any antifugal activity, it has a bacteriostatic and bactericidal activity, mainly against pathogenic Gram-positive bacteria (Suarez et al, 2003)

Alpha-helical

Linear antimicrobial peptides that can assume an active, amphipathic alpha helical structure are the most abundant and widespread in nature. Antimicrobial peptides from different source, or even within the same organism, that have evolved to act against distinct microbial targets in different physiological contexts, can show a marked variation in size, sequence and structure. Despite that, they meet two common and functionally important requirements:

a net positive charge that attracts them to the anionic microbial surface and the ability to assume amphipathic structure favoring the insertion into microbial membranes (Tossi et al, 2000; Giangaspero et al., 2001; Koczulla and Bals 2003).

The antimicrobial activity is based on several mechanisms. The initial binding is thought to depend on electrostatic interactions between the positively charged peptides and the negatively charged molecules at the surface of the target cell. A secondary step results in the modification of the biophysical properties of the membrane caused by direct interactions with the peptide. The membrane-active properties of AMPs have been analyzed by model systems demonstrating induction of leakage of artificial liposomes and the formation of ion-permeable channels (La Rocca et al, 1999; Zhang et al., 2001).

Three main mechanisms (see fig 2 in this section). have been suggested for peptide permeation of the membrane of the target cell: (i) a barrel-stave mechanism involves the formation of transmembrane channels in a voltage-dependent manner with non-polar molecule parts facing the membrane lipids and forming a hydrophilic pore spanning the membrane; (ii) the aggregate channel model involves aggregation of peptides in unstructured clusters in the membrane allowing the dynamic formation of pores for short time and the leakage of intracellular components. AMPs can also enter the intracellular space through this mechanism; and (iii) a carpet like mechanism describes the covering of the microbial cell membrane by a carpet of AMPs. The integrity of the membrane collapses by the formation of worm holes that form by the bending of the lipid layer back on itself.

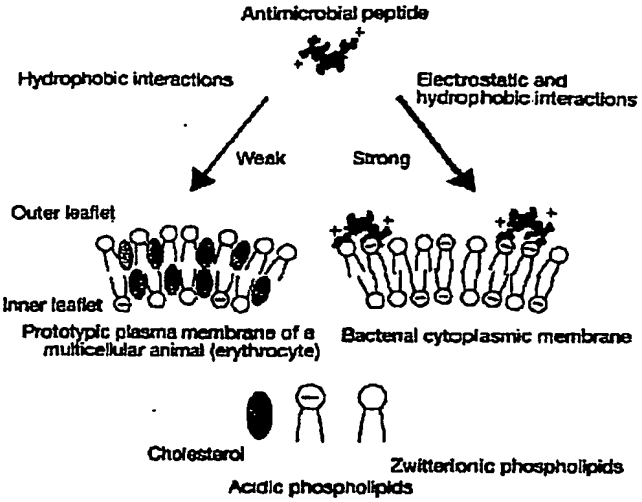
These interactions described in the three models are proposed to lead to loss of membrane function including breakdown of membrane potential, leakage of metabolites and ions and alteration of membrane permeability. The partial selectivity of AMPs for prokaryotic cells seems to depend on the different lipid composition associated with different amounts of negative charges of membranes of micro-organisms compared with eukaryotic cells. (Tossi et al, 2000; Giangaspero et al., 2001 ; Zasloff 2002 ; Koczulla and Bals 2003).

3.2 Aim

Since Flo polypeptide exhibit clarification and unexpectedly, disinfection properties we would like to identify the molecular basis and mechanism of each one of these two activities.

The next goal will be determining whether the whole polypeptide is important for the full activity or whether a common or distinct sequences would mediate both activities, thus suggesting similar or distinct molecular mechanism, respectively. To accomplish this approach we first modelized Flo structure based on homologies with a 2S seed protein. Based on the domains predicted by bioinformatic tools I synthesized several Flo subfragments.

Fig 1 Membrane target of antimicrobial peptides of multicellular organisms and the basis of specificity (From Zasloff, 2002)



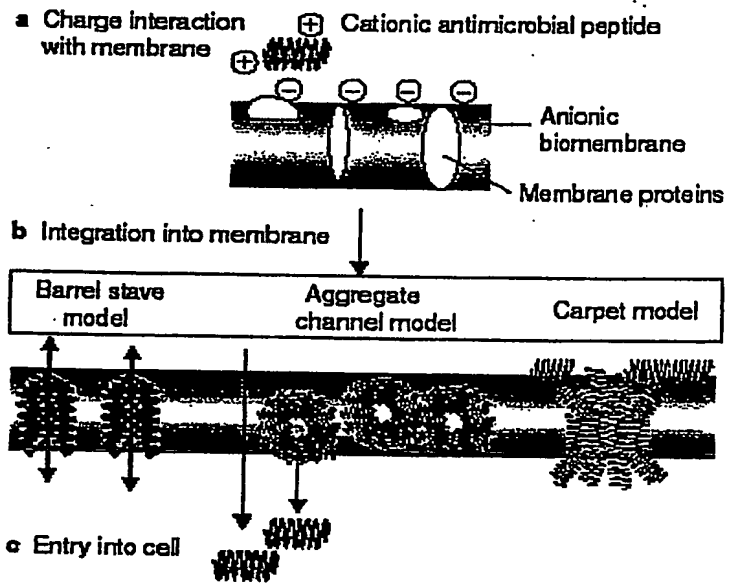


Figure 2 Antimicrobial activity of antimicrobial peptides (AMPs). AMPs accumulate near microbial membranes as a result of electrostatic interactions between negatively charged membranes and positively charged peptides (a). In a next step, the peptides associate with the membranes leading to a destabilization of the membrane and subsequent cell death of the microorganism (b). Several models of action may apply. Barrel stave model: helical, amphipathic peptides accumulate in the membrane as a barrel-like structure with non-polar molecule parts facing the membrane lipids and forming a hydrophilic pore spanning the membrane. Aggregate channel model: after binding to the membrane the peptides aggregate in clusters in the membrane with subsequent dynamic formation of pores for short time. AMPs can also enter the intracellular space through this mechanism. Carpet model: AMPs cover the microbial cell membrane in a carpet-like formation, which causes a collapse of the integrity of the membrane. In addition to membrane interaction, some AMPs enter the cell of the microorganisms and inhibit cellular processes such as RNA or protein synthesis (c).(from Koczulla and Bals, 2003)

3.3 Materials and methods

Synthetic polypeptides.

Synthetic Flo was synthesized at the Peptide-Protein Chemistry Facility of the University of Lausanne using the amino acid sequence of *Moringa oleifera* seed component M.O. 2.1 (Gassenschmidt et al., 1995). Synthetic polypeptides derived from the Flo sequence were synthesized using the Fmoc-chemistry procedure (Novabiochem, Germany) on an Abimed AMS 422 Multiple Peptide synthesizer (Germany) at the Laboratoire de Biotechnologie Chimique (EPFL). Cleavage and deprotection were performed employing the TFA protocol (Novabiochem, Germany) Finally, HPLC purification was performed employing a Xterra® Prep MS C18 5mm 10mm X 100mm (Waters Corporation, Mass, USA) or monolithic Chromolite™ Performance RP-18e column 4.6 X 100 mm (Merk, Germany). In all cases the mobile phase A was 0.1 trifluoroacetic acid in water and B was 0.085 trifluoroacetic acid in Chromasolv® acetonitrile (Riedel-deHaen, Seelze, Germany).
Mass spectrometry analysis was performed at the LBCH facilities on a LCT instrument (Micromass). This instrument combines an electrospray source and a Time of Flight (TOF) analyzer. The peptide samples were diluted in a final mixture of water/acetonitrile (1/1) and sprayed at a flow rate of 10 microlitres/min. The mass spectrometer was tuned according to the size of the product of interest but with a broad range in order to allow the identification of contaminants. External calibration was performed to allow accurate mass measurement.

Computational analysis of primary sequence and secondary structure

Flo polypeptide primary accession number is P24303 (Gasteiger et al, in press). After a first scanning searching for homologies, analyses of secondary structure were performed employing consensus prediction programs (Network Protein sequence analysis, Pole Bio-informatique lyonnais ; PSIPRED. UCL Bioinformatics Unit, Department of Computer Science, University College London; Jpred2 Barton Group, EMBL-European BioInformatics Institute).

CD Spectroscopy

It was measured in a Jasco 710 CD spectropolarimeter (Institute for Molecular Biology and Biophysics ETHZ, Zurich), employing quartz cuvettes with 1 mm path length. CD spectra were usually accumulated 8 times and averaged in the far UV. All measurements were

performed in 50mM phosphate buffer pH 7.0 without trifluoroethanol to mimic the conditions of Flo activity. Urea concentration ranged from 0.03 to 8M.

3.4 Results

Computational analysis of primary and secondary structure

Amino acid sequence analysis indicated similarities between Flo and other proteins belonging to the 2S albumin seed protein family. 2S proteins are initially synthesized as a precursor protein, which is proteolytically cleaved to generate mature chains: a bigger one (B chain, 9000 Da) and a smaller one (A chain, 4000 Da) held together by disulphide bonds. In this precursor protein, the A subunit corresponds to the N terminal region meanwhile the B subunit corresponds to the C terminal one (Josefsson et al, 1986 ; Ferl et al., 1983) Sequence comparisons have revealed that one member of this 2S protein family, called napins, belong to a more diverse protein family, which includes major allergens, trypsin inhibitors and natural antifungal proteins. Both subunits have been crystallized and have been shown to contain several alpha helices (three in the B subunit and two in the A subunit of napins).

When aligning Flo sequence with the one of napin and mabinlin it can be observed that Flo has specifically a significant identity with the 8 kDa heavy chains of two members of this family, moreover the N-terminal portion of the polypeptide present in the napins and mabinlins is missing in Flo (fig 1). Flo may therefore represent a truncated version of the native *Moringa oleifera* seed 2S protein. As expected from this finding, bacterial and synthetic Flo migrated just below the heavy chain of the seed extract major protein (Suarez et al., 2003).

Secondary structure predictions based on amino acid sequence alignment between Napin heavy chain and Flo amino acid sequence indicate the presence of three putative alpha helices in Flo. This secondary structure model of Flo arose equally when employing consensus prediction programs. In all cases the sequence showed a high probability of alpha helices in three specific regions (fig. 2) (Rice et al, 2000 ; Gasteiger et al, in press). Four polypeptides (fig 3) representing partial fragments from Flo, that correspond to each one of the putative alpha helices, were synthesized and named polypeptide 1 (P1), 2 (P2), 3 (P3). Another polypeptide (P2.1) was sinthetized later. Both synthetic Flo and Flo derivatives polypeptides have the same purity, as verified through the ASA assay or HPLC profiles.

Coagulation assays.

Efficient coagulation was observed with saturating amounts of P2 at the same levels as chemically synthesized Flo. Moreover, both of them exhibit comparable activities at subsaturating amounts in this semiquantitative assay (fig. 4) loosing detectable activity

between 0.1 and 0.05 mg/ml. This suggests that P2 is sufficient to produce the same effect as the whole Flo polypeptide. On the other hand, no activity was observed neither for P1 nor for P3, even at 50 mg/ml (data not shown).

A

```
QSGPQQGPWLREQCCNELYQEDQVCV- CPTLKQAAKSVRV-- --QGQHGPFQSTRI--YQI--- AKNLP (N)
   GP     P     ++ C    + L             CP+L+QA              QGQ  GP  Q       Y+  A N  P
 QGPGRQPDF---QRCGQQLRNISPPQR -CPSLRQAVQLTHQQ ---QGQV GPQQVRQMYRVASNI P (F)
   GP              C    QLR             CP LRQA      HQQ   QGQ   GP  QVRQ    R A N  P
EQRGPALRL---------CCN-- QLRQVNKPCVCPVLRQAA------HQQLYQGQIEGPRQVRQLF- RAARNLP (M)
```

B

Napin, B chain (rape: *Brassica napus*, 75aa)

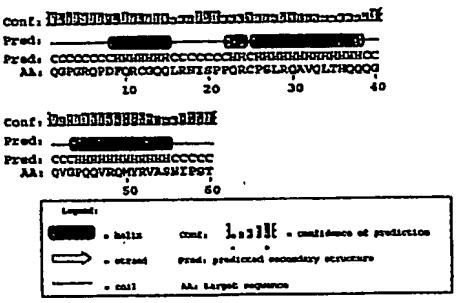
QSGPQQGP REQCCNELYQ EDQVCVCPTL KQAAKSVRVQ GQHGPFQSTR IYQIAKNLPN VCNMKQIGTC PFIAI

Mabinlin I-1, B chain (tropical raiforest plant: *Capparis masakai*, 72aa)

EQRGPALRLC CNQLRQVNKP CVCPVLRQAA HQQLYQGQIE GPRQVRQLFR AARNLP NICK IPAVGRCQFT RW

Figure 1 Homology among Napin, Flo and Mabinlin amino acid sequences
Panel A: here it is indicated the region of homology among Flo (F), Napin (N) and Mabinlin (M). The name of the amino acid between the sequences indicates identity, " +" indicates conservative changes Only partial sequences of Napin and Mabinlin is shown. The identity between N and F is 32%; between M and F is 47% and between N and M is 28%. Penalties due to the gaps included are not considered.
Panel B: The above mentioned region of homology (underlined) does not comprise the C terminal region of Napin or Mabinlin

A



B

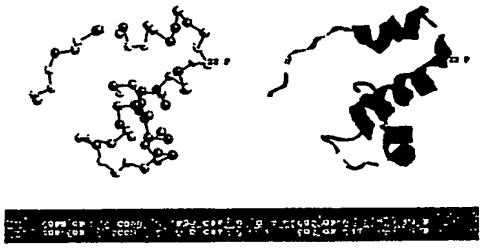


Figure 2 Predicted alpha-helices on Flo sequence (A) Schematic representation of Flo secondary structure; (B) computer generated structure of Flo based on the crystal structure of Napin The alpha helices are separated by one turn and by one loop. It is indicated the proline at position 22

Flo polypeptide

QGPGRQPDFQRCGQQLRNISPPQRCPSLRQAVQLTHQQQGQVGPQQVRQMYRVASNIPST

QGPGRQPDFQRCGQQLRNISPP GQVGPQQVRQMYRVASNIPST

PQRCPSLRQAVQLTHQQQGQV

Polypeptide 1 Polypeptide2 Polypeptide 3

CGQQLRNISPPQRCPSLRQAVQLTHQQQGQV

Polypeptide 2.1

Fig 3. Flo subfragments corresponding to the putative alpha helices P1 stands for the N terminal end of Flo (21 aa) P2 the middle part (21 aa) and P3 the C terminal end (22 aa length). P2.1 polypeptide comprises P2 and half of P1

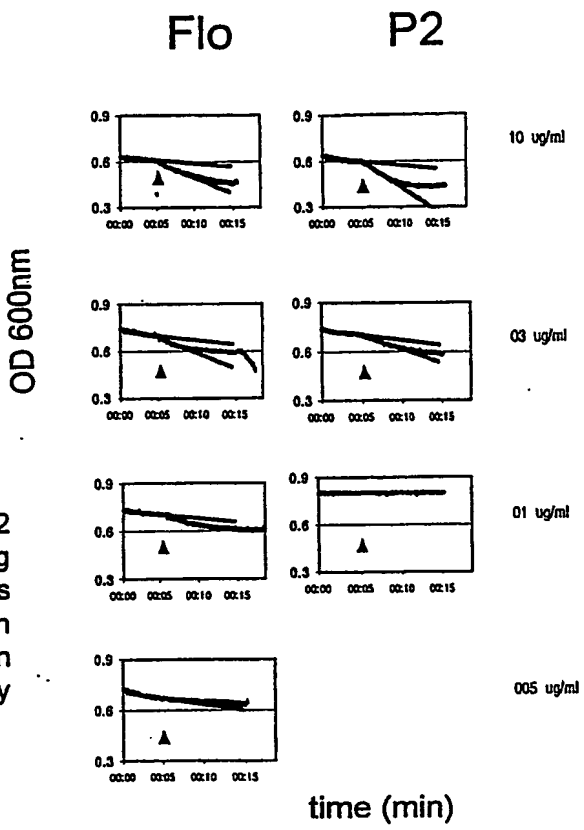


Fig 4. Coagulation activity of Flo and P2 Titration experiment with subsaturating amounts of polypeptide 2 (three panels at the right column) and Flo (four panels on the left column) following the coagulation test procedure described previously

mg/ml	Flo	P1	P2	P3	P2.1
0	100	100	100	100	100
0.2	$6.5X10^{-2}$	367	27.5	271	13.9
2	$<10^{-3}$	74	10	695	$<10^{-3}$
6	$<10^{-3}$	1	$2.5X10^{-3}$	3.6	$<10^{-3}$

Table 1 Effect of Flo and Flo derivative polypeptides on *E. coli*
Firstly, cell suspensions were incubated 0.2, 2 and 6 mg/ml of polypeptide, then they were washed with one volume of phosphate buffer 10mM pH=7.0 to remove the polypeptide from the medium. Finally, they were spread on non-selective solid media Cell viability is expressed as percentage of colony forming unit after treatment. 100% of survival is the colony forming unit number in absence of polypeptide (1 to $5{\cdot}10^9$ CFU/ml)

Polypeptide antibacterial effect

Among the three Flo derived polypeptides tested, P2 is the most efficient in inhibiting the growth of *E. coli*. Table 1 shows that P3 is the polypeptide that exhibits the lowest level of antimicrobial activity, cell culture growths in presence of 0.2 and 2mg/ml. It is only inhibited when the concentration reaches 6mg/ml. When comparing P1 and P2, it can be observed that both of them exert and inhibitory effect. However this effect is stronger for P2, nevertheless, none of them are as efficient as Flo in terms of antimicrobial activity.
Since P2 and P1 were the most active ones, a fourth polypeptide was synthesized. Named P2.1, this peptide comprises the full length P2 with the C terminal half of polypeptide P1 (fig 1). Preliminary results showed in table 1 indicate that P2.1 shows an antimicrobial activity comparable to that of Flo.

CD Spectroscopy

CD profile is likely to show that Flo has a coiled structure under the conditions employed for the water coagulation test (10mM phosphate buffer, pH=7.0). Since it is known that conformational transitions are enhanced under increasingly denaturing conditions, urea was added at increasing concentrations (0.03M-8M) in the CD assay in order to observe the effect on the Flo secondary structure. However, since the highest concentrations of urea used have a strong interference at the far UV spectra, the obtained data did not add to clarify the conformational analysis (data non shown).

3.5 Discussion

Despite that Flo may not comprise a full B subunit length protein, it exhibits coagulation and antibacterial activity. However, when performing the experiences with the Flo derivatives polypeptides, they showed full, partial or no activity at all.
Polypeptides 1, 2 and 3 were synthesized based on the criteria that each might represent a different putative Flo alpha helix.

Coagulation mechanism

Regarding the flocculation activity, P2 is as active as Flo. Since P2 does not have more positive charges than the other two polypeptides (two arginine in P2, three in P1 and two in P3). Other feature(s) might be responsible for its higher coagulation activity. The three dimensional model predicts the longest alpha helix for P2, however it is not clear whether this secondary structure is essential for this activity. On the contrary, CD studies indicated that Flo has a random coil structure.
As was previously mentioned, the maximum turbidity reduction *Moringa oleifera* seed extract corresponded to a zero zeta potential. This could be interpreted as the adsorption and neutralization of particles, a mechanism that corresponds to a coagulant agent (Ndabigengesere et al, 1995). However if we consider that Flo derivative polypeptides may have a random coil structure it cannot be excluded the patch charge mechanism. In this latter one positively charged proteins bind to negative charged particles leading to a neutralization of charges and/or formation of flocs (Eisenlauer and Horn, 1985)

Antibacterial mechanism

When tested for their antibacterial capacity, none of the three polypeptides seems to efficiently replace the activity displayed by Flo. Moreover, previous results indicated a hierarchy of activity among the different polypeptides: Flo>P2>P1>P3, suggesting that most of the antibacterial activity is contained in the P2 and P1 sequences. Consequently, a new polypeptide (P2.1) containing the entire P2 sequence with the adjacent half of P1 was synthesized and its antimicrobial activity tested. Results show that P2.1 polypeptide has a similar level of antibacterial activity to that of full-length synthetic Flo.
In the polypeptide P2.1, the fragment of P1 is linked to the fragment corresponding to P2 through two prolines (position 21 and 22) thereby favourising the formation of a turn between the two elements. Perhaps the maintenance of this structural feature might be essential for the antibacterial activity.

The relatively short, linear antimicrobial peptides considered here are generally unstructured in solution. However in many cases these polypeptides gain structure when they interact with their target membranes (Moreman et al, 2003). Preliminar results indicate the same for Flo (Hovius R, unpublished results) .

Being cationic, these peptides are electrostatically attracted to negatively charged microbial surfaces. In the case of gram negative bacteria, they interact initially with the lipopolysaccharide (LPS)-rich external leaflet of the outer membrane, which they can traverse by displacing magnesium ions and disrupting non covalent bridging interactions between LPS molecules, thus altering the ordered supramolecular arrangement of this leaflet. The surface of gram-positive bacteria is negatively charged due to the presence of teichoic and teichuronic acids, and of amino acid carboxyl groups in the multilayered peptidoglycan, which they have to traverse. Once the microbial cytoplasmic membrane is reached, peptides initially interact with the negatively charged phospholipid head groups of the external leaflet, then they assume an amphipathic helical conformation that allows them to insert the hydrophobic face into the bilayer.

This interplay of initial electrostatic interaction followed by hydrophobic partitioning confers on antimicrobial peptides the unique characteristic of being highly water soluble and yet able to interact strongly with phospholipids bilayers.

Conclusion

Other important parameters that have to be taken into account to better understand the function of the peptide is the position-dependent hydrophobicity and the glutamine tracts.

Hydrophobicity calculations indicate that the region of P2 comprising the amino acidic sequence RQAVQ might be the most hydrophobic (values above 1.0; Kyte-Doolittle scale). This is important for the interaction between lipids and peptides.

Regarding glutamine tracts, this residue represent 25% of the whole Flo polypeptide. Glutamine is involved in protein oligomerization through the formation of some β-sheet structure (Stott et al. 1995). Among P1, P2 and P3. the fragment corresponding to P2 is the one that exhibits more of this residue (seven).

From all these results, it is likely that the fragment corresponding to P2 is the most active one, being enough for water coagulation. However, in the case of the antibacterial, a longer polypeptide is required comprising part of P1 polypeptide. Thus, the mechanisms that are involved in coagulation and antibacterial effect are likely to depend on different parameters.

3.6 Perspectives

Since the ultimate goal will be producing an improved molecule it will be important to define the parameters involved in the interaction between the polypeptides and their targets. One approach will be to define the shortest active amino acid sequence both for coagulation and antimicrobial activity. Narrowing of the amino acid sequence should improve the understanding of the mechanism involved. It would also be important to identify conformational transitions either on Flo or on its derivatives polypeptides (particularly P2 and P2.1). For this purpose they will be incubated in presence of glass beads or lipids layers mimicking thus, the natural interactions. Punctual amino acid mutations will also help because they would modify the polypeptide structure in a controlled manner (replacing for example charged or hydrophobic amino acids). This mutations will be defined based on bioinformatic tools and circular dichroism results.

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3.8 Acknowledgements

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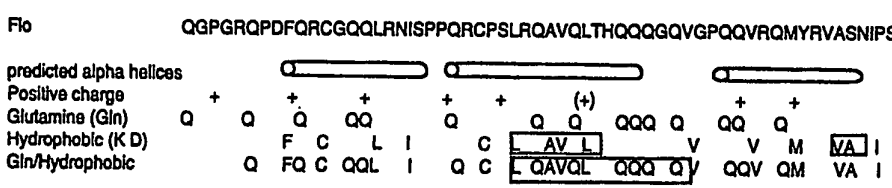
Scheme of Flo and derivate peptides employed in this study



		Coag. act.	antibact. act.
P1	QGPGRQPDFQRCGQQLRNISPP	–	+/-
P2	PQRCPSLRQAVQLTHQQQGQV	++	+
P3	GQVGPQQVRQMYRVASNIPST	–	–
P2a	PQRCPSLRQAV	–	ND
P2b	SLRQAVQLTHQ	–	ND
P2c	AVQLTHQQQGQV	–	ND
P2ab	PQRCPSLRQAVQLTHQ	++	ND
P2bc	SLRQAVQLTHQQQGQVP2.1G40R	–	ND
P2.1	RCGQQLRNISPPQRCPSLRQAVQLTHQQQGQ	–	++

Near future	
P2abab	$P(QRCPSLRQAVQLTHQ)_2$
P2.1G40R	RCGQQLRNISPPQRCPSLRQAVQLTHQQQRQ
P2.3	PQRCPSLRQAVQLTHQQQGQVGPQQVRQMYRVASNIPST
P1.2	QGPGRQPDFQRCGQQLRNISPPQRCPSLRQAVQLTHQQQGQV

This scheme summarizes all the polypeptides employed in this study at present time. In all cases, the adopted strategy is to make shorter fragments with the aim to identify the shortest active polypeptide.

First, Flo amino acid sequence is shown. Next, its relevant structures that might be important for its activity (alpha helices, charges, glutamine and hydrophobic regions –see chapter 3 of half thesis rapport-). The positive charge of histidine (H) is facultative since it depends on the pH of the environment (below pH=6.75 it is charged). Glutamine amino acid is indicated since literature remarks that this residue might be implicated in peptide multimerization. For hydrophobic characterization (Kyte-Doolittle scale) regions with overall values above 0 are hydrophobic in character and this corresponds to the two indicated rectangles The last line (Gln/Hydrophobic) indicates a relevant cluster that might be important for the activity. At the right of the scheme, two columns show the coagulation activity and antibacterial activity, respectively. Flo represent the peptide with the maximal activity, indicated as «++»; ND means «not determined» .

Polypeptides P1, P2 and P3 correspond to the length where each one of the alpha helices from Flo should span. Subsequent results showed that most of the activity is located in P2, so several subfragments of P2 were synthesized to localize more precisely the active portion: P2a, P2b and P2c, as well as P2ab and P2bc.

Due to the fact that some antibacterial activity is already lost when employing P2, a longer polypeptide was created (P2.1) allowing the recovery of the original activity. At present the best polypeptide for the coagulation activity is P2ab and for the antibacterial activity is P2.1

In the near future I will use other variations of the optimal polypeptides trying to improve their activity. For this purpose we will synthesize P2.1G40R (replacing a glycine by an arginine at position 40) increasing the whole polypeptide charge by one, and P2abab (a tandem of P2ab). Furthermore, to complete the scenario it will be important to further characterize the coagulation and antimicrobial activity of P2.3 and P1.2

Summary of some Flo derivatives

	Coag.	antibt.
QGPGRQPDFQRCGQQLRNISPPQRCPSLRQAVQLTHQQQGQVGPQQVRQMYRVASNIPST	++	++

predicted alpha helices
XXXXXXXXXXXXXX XXXXXXXXXXXXXXXXX XXXXXXXXXXXXXX

Positive charge
+ + + + + (+) + +

Glutamine (Gln)
Q Q Q QQ Q Q Q QQQ Q QQ Q

Hydrophobic
F C L I C L AV L V V M VA I

Gln/Hydrophobic
Q Q FQ C QQL I Q C L QAVQL QQQ Q V QQV QM VA I

P1 QGPGRQPDFQRCGQQLRNISPP	-	+/-
P2 PQRCPSLRQAVQLTHQQQGQV	++	+
P3 GQVGPQQVRQMYRVASNIPST	-	-
P2.1 RCGQQLRNISPPQRCPSLRQAVQLTHQQQGQ	-	+++
P2a PQRCPSLRQAV	-	-
P2b SLRQAVQLTHQ	-	-
P2c AVQLTHQQQGQV	-	-
P2ab PQRCPSLRQAVQLTHQ	++	+/-
P2GR40 PQRCPSLRQAVQLTHQQQRQV	+++	++

Introduction

Problems in reproducibility of the coagulation test was noted for several Flo subfragments, notably P2. It was observed solubility problems, raising the possibility that lack of proper solubility was causing inconsistencies

GOAL

Solve reproducibility problems by improving the solubility by lowering pH

1- Coagulation test: effect of pH on peptide activity

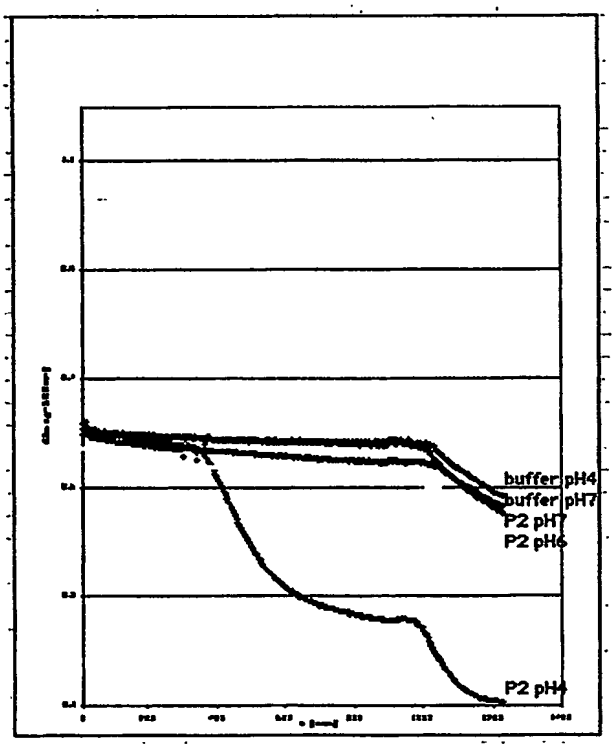
2- Antimicrobial test: effect of pH on peptide activity

1- Effect of pH on peptide activity: coagulation test

P2 is positively charged with some hydrophobic residues. We tried to improve solubility by lowering the pH of the resuspending solution (pH 7, then 6 & finally 4)

The assay was performed as usual in 10mM Ph buff pH 7.0

Until now, all the polypeptides were used at 10ug/ml (1.47uM Flo). However, from now, instead of employing the same concentration, what is going to be taken into account is the molarity (1.47uM).



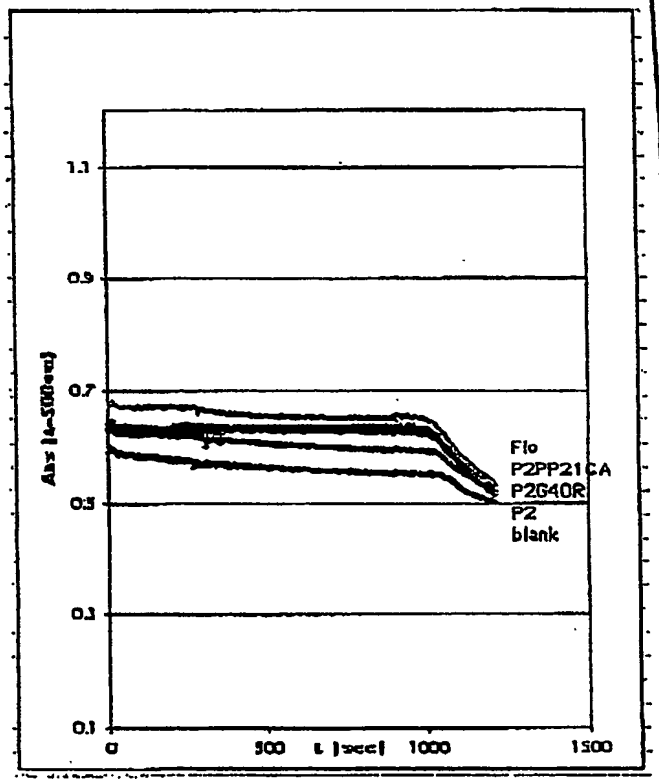
Several different pH

P2: 1.47uM

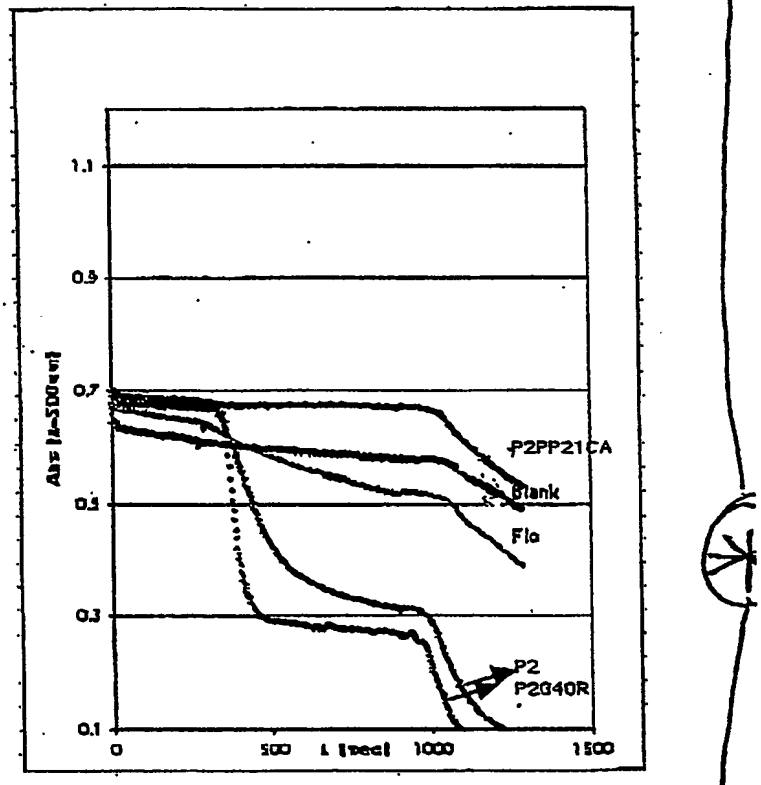
Polypeptide 2 sedimentation efficiency improves dramatically when it is diluted in acid pH

Comparison of equivalent molarities of Flo, P2 and derivatives

After the previous result, all the polypeptides were tested again
the molarity among them was always taken into account (1.47uM) which corresponds to 10ug/ml of Flo and aprox 3ug/ml of P2



pH 7 / 1.47uM



pH 3 / 1.47uM

demonstration that P2G40R has better flocculation activity than Flo

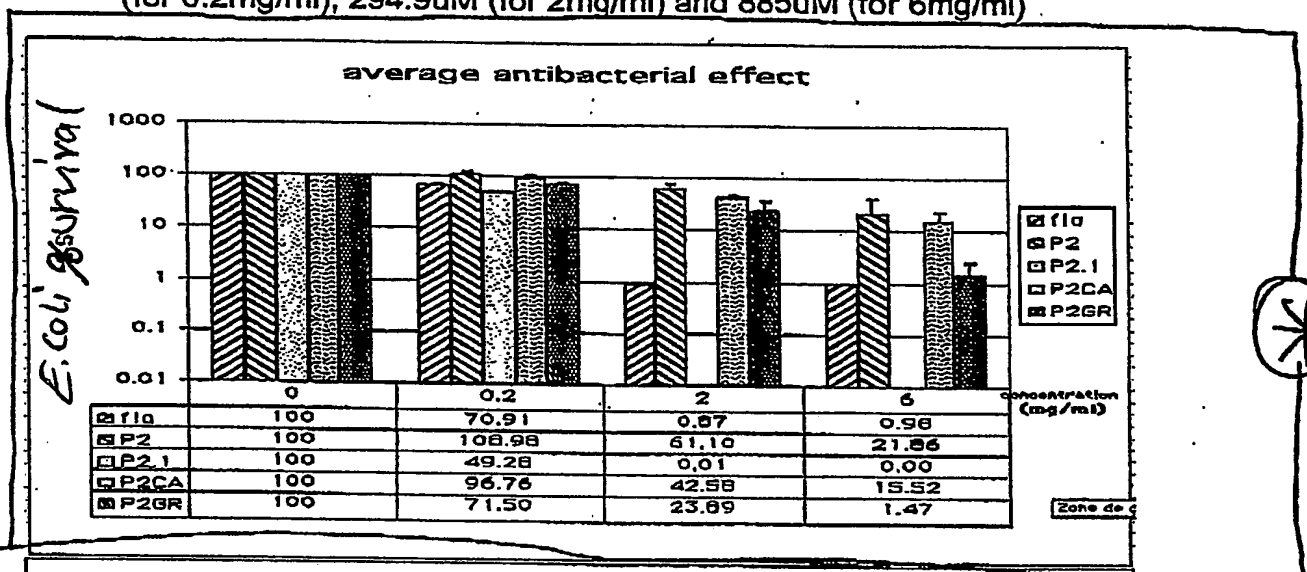
The specific activity (sedimentation efficiency/uM) is better for P2 as compaired to Flo. An improved efficiency (better than P2) is also observed for the P2G40R. In all cases P2PP21CA is less active than both of them

2- Effect of pH on peptide activity: antimicrobial test

Since pH of the solubilizing buffer has a drastic effect on the coagulation efficiency, it was interesting to know whether this was the same for the antimicrobial test

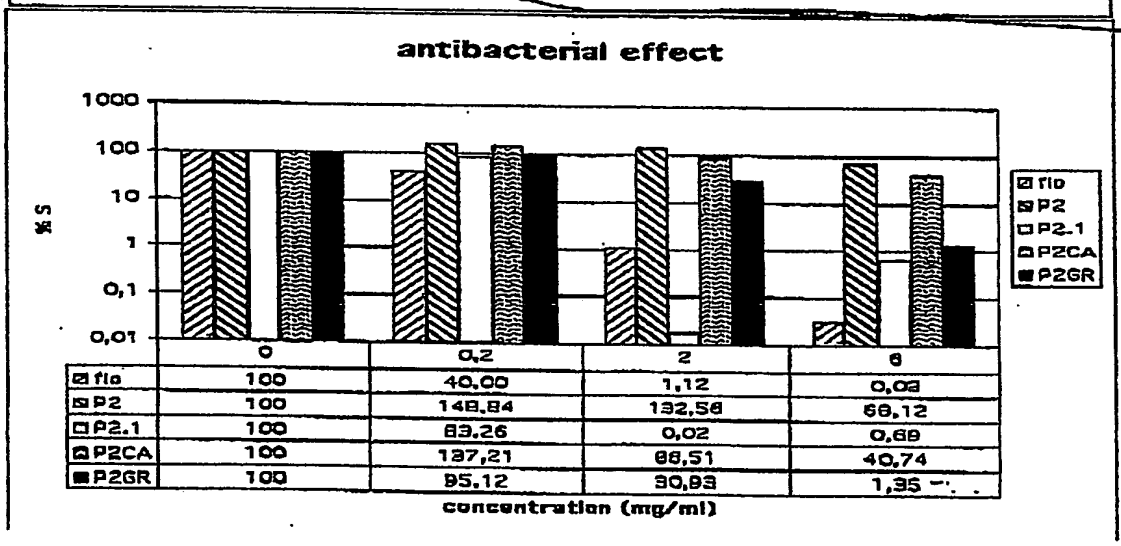
Conditions: same molarity for all the polypeptides taking Flo as a reference: 29.49uM (for 0.2mg/ml), 294.9uM (for 2mg/ml) and 885uM (for 6mg/ml)

Isomolar pH=7



	0	0.2	2	6
flo	100	70.91	0.87	0.98
P2	100	108.98	61.10	21.86
P2.1	100	49.28	0.01	0.00
P2CA	100	96.76	42.58	15.52
P2GR	100	71.50	23.89	1.47

Isomolar pH=4



	0	0,2	2	6
flo	100	40,00	1,12	0,03
P2	100	148,84	132,56	68,12
P2.1	100	89,26	0,02	0,68
P2CA	100	137,21	88,51	40,74
P2GR	100	95,12	30,93	1,35

The antibacterial effect does not change significatively with the pH,

P2.1 is the most active peptide irrespective of the pH

This result confirms that Flo = P2.1 > P2G40R > P2 > P2PP21CA

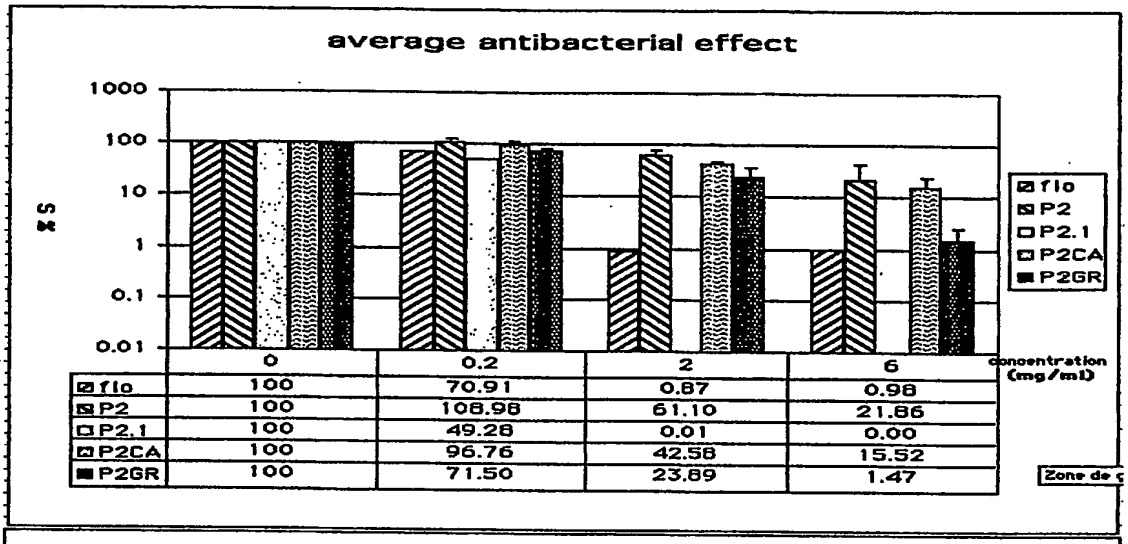
* demonstration that P2.1 can display more antibacterial activity than Flo.

2- Effect of pH on peptide activity: antimicrobial test

Since pH of the solubilizing buffer has a drastic effect on the coagulation efficiency, it was interesting to know whether this was the same for the antimicrobial test

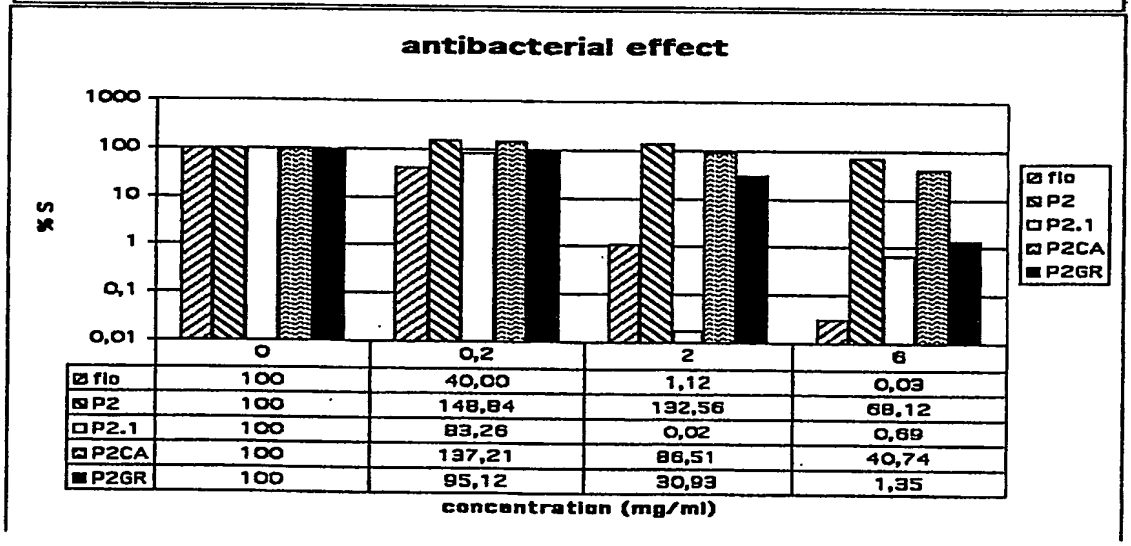
Conditions: same molarity for all the polypeptides taking Flo as a reference: 29.49uM (for 0.2mg/ml), 294.9uM (for 2mg/ml) and 885uM (for 6mg/ml)

Isomolar pH=7



	0	0.2	2	6
flo	100	70.91	0.87	0.98
P2	100	108.98	61.10	21.86
P2.1	100	49.28	0.01	0.00
P2CA	100	96.76	42.58	15.52
P2GR	100	71.50	23.89	1.47

Isomolar pH=4



	0	0,2	2	6
flo	100	40,00	1,12	0,09
P2	100	148,84	132,56	68,12
P2.1	100	83,26	0,02	0,69
P2CA	100	137,21	86,51	40,74
P2GR	100	95,12	30,93	1,35

The antibacterial effect does not change significatively with the pH, P2.1 is the most active peptide irrespective of the pH

This result confirms that Flo = P2.1 > P2G40R > P2 > P2PP21CA

RESULTS SUMMARY AND CONCLUSIONS

Regarding flocculation activity:

P2 and P2G40R are likely to be more soluble at acid pH, yielding extremely high activity for P2G40R.
Neither P1 nor P2.1 are active. Moreover, none of the P2 subfragments show activity, except for P2ab.
P2ab is the smallest active fragment (16aa), comprising positively charged amino acidic residues and a hydrophobic-glutamine patch
P2a and P2b are inactive, we hypothetize that it could be due to the fact that these two polypeptides have only one of such domains. Perhaps the positive charged arginines are involved in coagulation and the hydrophobic+glutamine patch in flocculation

P2	PQRCPSLRQAVQLTHQQQGQV
P2ab	PQRCPSLRQAVQLTHQ
P2a	PQRCPSLRQAV
P2b	SLRQAVQLTHQ

Regarding antibacterial activity:

pH seems not to have an effect on this activity
Results confirm that P2 G40R is more efficient than P2 and PP21CA, suggesting that the increased positive charge and/or amphipathy improves activity
P2.1 remains most active peptide

Acknowledgements to Patrice de Werra (4th year biology student)

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Claims

1. Active fragment of polypeptide FLO as defined in the description.

2. Use of FLO or an active fragment of it in the manufacture of a drug against Helicobacter Pilori

Abstract

Active fragment of polypeptide FLO as defined in the description.